UNIVERSIDADE FEDERAL DO PARANÁ

GUSTAVO HENRIQUE QUEIROZ SCHUNEMANN MANFRIN DE OLIVEIRA

EFEITOS DA GALANTAMINA NO COMPROMETIMENTO DA MEMÓRIA E AUMENTO DE BETA-AMILÓIDE E ALFA-SINUCLEÍNA NO MODELO ANIMAL DE DOENÇA DE ALZHEIMER INDUZIDO POR ESTREPTOZOTOCINA.

CURITIBA

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Orientadora: Profa. Dra. Maria Aparecida Barbato Frazao Vital.

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NOTA EXPLICATIVA

Esta dissertação é apresentada em formato alternativo – artigo para publicação – de acordo com as normas do Programa de Pós-Graduação em Farmacologia da Universidade Federal do Paraná.

RESUMO

O modelo de doença de Alzheimer (DA) induzida por Estreptozotocina (STZ) gera um estado de resistência cerebral à insulina, capaz de causar prejuízo cognitivo nos animais que se assemelha aquele observado na DA, devido ao estresse oxidativo mediado por processo inflamatório. Os níveis de citocinas inflamatórias podem ser reduzidos pelo tratamento com Galantamina, uma vez que esse fármaco pode interferir no metabolismo glicídico; assim, seu uso é indicado para amenizar os sintomas da DA uma vez que a deficiência no metabolismo da glicose pode participar da patogênese da DA. Neste contexto, foram investigados os efeitos da Galantamina na memória e frequência de locomoção de ratos Wistar tratados com STZ. Para isso, mimetizamos a DA em ratos Wistar machos por meio de injeções intracerebroventriculares de STZ (1mg / kg). Em seguida, testes comportamentais foram realizados em 32 ratos: 16 injetados com STZ e 16 injetados com veículo. Posteriormente, metade dos ratos em cada um dos grupos foi tratado com Galantamina (5 mg / kg). A outra metade recebeu veículo. Além disso, realizamos teste tipo ELISA para avaliar o acúmulo de Alfa Sinucleína (α-Syn) e Beta Amilóide (Aβ) nos cérebros dos ratos. Os resultados de alguns testes comportamentais indicaram que o grupo STZ + Veículo possuía prejuízos cognitivas, quando comparado ao grupo Sham + Veículo. Não houveram diferenças estatisticamente significantes na memória dos ratos tratados com Galantamina, quando comparados aos tratados apenas com veículo. O ELISA mostrou acúmulo de Aβ e α-Syn em grupos de ratos lesados pela STZ. Este último resultado sugere que o comprometimento da locomoção encontrado nos animais pode ser devido ao acúmulo de α-Syn e não apenas pela mimetização de DA causada pela STZ.

Palavras-chave: Doença de Alzheimer. Galantamina. Estreptozotocina. Alfa Sinucleína. Beta Amilóide.

ABSTRACT

The Streptozotocin (STZ) induced animal model of Alzheimer's Disease (AD) generates a state of cerebral insulin resistance, capable of mimicking AD by causing oxidative stress mediated by inflammatory process. The levels of inflammatory cytokines can be reduced by Galantamine treatment; thus, its use is indicated to ease AD symptoms, since it is believed that deficiency in glucose metabolism may participate in the pathogenesis of AD. In this context, we aim to investigate the effects of Galantamine regarding memory and locomotion frequency in Wistar rats treated with STZ. To do so, we mimic the AD in male Wistar rats through intracerebroventricular injections of STZ (1mg/kg). Then, behavioral testes were carried for 32 rats: 16 injected with STZ and 16 injected with vehicle. Subsequently, we treated half of the rats in each of the aforementioned groups with Galantamine (5 mg/kg). The other half of the rats of each group were given vehicle. In addition, we performed ELISA to assess Beta Amyloid (Aβ) and Alpha Synuclein (α-Syn) accumulation in the rats' brains. Except for the Novel Object Recognition Test, all other behavioral tests results indicated that the STZ + Vehicle group was impaired when compared to the Sham + Vehicle group. In addition, our results showed no statistically significant differences in the memory of the rats treated with Galantamine, when compared to those only treated with vehicle, regarding behavioral tests. The ELISA showed Aβ and α-Syn accumulation in some groups of rats impaired with STZ. This last result suggests that the locomotion impairment found in the animals could be due to the α-Syn accumulation and not only because of the AD mimicking caused by the STZ. Finally, the animals impaired with STZ did not presented improvements when treated with Galantamine, nonetheless, the overall performance for the STZ + Galantamine group was higher than the STZ + Vehicle one on the NORT. Hence, we believe that more studies on this subject should be carried out to elucidate some challenges yet unresolved.

Keywords: Alzheimer's Disease, Galantamine, Streptozotocin, Alpha Synuclein, Beta Amyloid.

SUMÁRIO

1 INTRODUÇÃO

Demência é a principal causa de distúrbios progressivos em funções cognitivas como linguagem, memória, pensamento racional e habilidades sociais, além disso, ela é capaz de promover alterações emocionais e de personalidade (Cipriani *et al.*, 2015). Um dos tipos mais comuns de demência é a doença de Alzheimer (DA). Atualmente, estima-se que 35,6 milhões de pessoas vivam com ela e acredita-se que essa estimativa possa dobrar a cada 20 anos, chegando a 65,7 milhões em 2030 (Dos Santos *et al.*, 2018).

Por ser uma doença neurodegenerativa progressiva de caráter irreversível, a DA afeta a cognição e interfere na memória, o que leva o indivíduo à demência total em estágios avançados. No entanto, a doença também pode interferir nas capacidades físicas de pessoas afetadas (memória, cognição, fala e funções motoras), o que a torna a principal causa de dependência funcional, institucionalização e mortalidade na população idosa (Dos Santos *et al.*, 2018; Silvestrelli *et al.*, 2006).

A neuropatologia da DA segue um padrão semelhante a outras demências, existindo um certo arquétipo seguido pelas células neurais que são acometidas: lesão neuronal, falha sináptica e finalmente morte celular (Kamat *et al.*, 2016; Lowe *et al.*, 2018).

Esses achados levam às duas hipóteses principais de alterações cerebrais causadas pela DA: a hipótese da cascata amilóide e a hipótese colinérgica. Ambas as hipóteses sugerem que o desfecho final do da DA é a perda funcional com a consequente morte neuronal, pelo acúmulo de proteína Beta Amilóide (Aβ) e emaranhados TAU (Barage e Sonawane, 2015; Pákáski e Kálmán, 2008; Sereniki e Vital, 2008).

Outra proteína potencialmente nociva é a α-sinucleína (α-Syn). Seu acúmulo pode levar a um processo de envelhecimento errôneo, através de corpos de Lewy, culminando em doenças neurodegenerativas como a doença de Parkinson (DP) e a doença do corpo de Lewy (Spillantini e Dickson, 2003). Embora a α-Syn seja comumente considerada um biomarcador de DP (Chang *et al.*, 2020), alguns estudos

demonstram que até 60% dos pacientes com DA apresentam sinais de distúrbios causados por corpos de Lewy (Arai *et al.*, 2001; Hamilton, 2000; James *et al.*, 2012; Schneider *et al.*, 2009). Em um estudo de coorte com 225 pacientes com DA, foi demonstrado que a concentração de α-Syn no líquido cefalorraquidiano estava significativamente aumentada (Majbour *et al.*, 2017). Outro estudo sugere que os distúrbios da DA tenham ligação com lesões simultâneas mediados por α-Syn (Mikolaenko *et al.*, 2005). Apesar de serem diferentes os possíveis desfechos clínicos oriundo dos acúmulo de Aβ e α-Syn, ambas proteínas compartilham mecanismos que levam ao estresse oxidativo (Angelova e Abramov, 2017).

Estudos sugerem que a diminuição do metabolismo neural da glicose desencadeia a cascata neurodegenerativa da DA (De La Monte e Wands, 2008; Grieb, 2016; Steen *et al.*, 2005). A partir disso, a Estreptozotocina (STZ), uma droga capaz de induzir o estado cerebral resistente à insulina (IRBS) (Barilar et al., 2020), pode ser usada para mimetizar algumas características clínicas da DA, quando administrada por via intracerebroventricular (ICV-STZ).

Apesar da literatura evidenciar conexões entre a proteína Aβ e a DA, ainda é necessário um melhor entendimento da relação entre a DA e a α-Syn. Até onde os autores desse trabalho tem conhecimento, não foram encontrados na literatura estudos sobre a relação entre a STZ, Aβ e α-Syn. Dessa forma, decidimos por investigar este assunto de maneira mais aprofundada neste trabalho.

Um crescente acúmulo de evidências sugere efeito neuroprotetor da Galantamina (Bloniecki *et al.*, 2017; Geerts, 2005; Geerts *et al.*, 2005; Wang *et al.*, 2019). A Galantamina possui propriedades antioxidantes, antiapoptóticas e antiinflamatórias, de tal maneira que pode modular o metabolismo da glicose (Ali, 2015; El-Abhar *et al.*, 2015).

A dose comumente usada em ratos varia de 1 mg / kg a 5 mg / kg (Furukawa *et al.*, 2014; Geerts, 2005; Geerts *et al.*, 2005; Satapathy *et al.*, 2011). Nesse cenário, um estudo demonstrou que ratos recém-nascidos pré-tratados com Galantamina tiveram redução nos danos cerebrais induzidos por hipóxia-isquemia. Neste estudo, a dose de 5 mg / kg de Galantamina mostrou acentuada diminuição de danos cerebrais quando comparada ao grupo controle e aos grupos pré-tratados com 1 e 2,5 mg / kg de Galantamina (Furukawa *et al.*, 2014).

Embora muito já se tenha sido elucidado acerca desse assunto, mais estudos sobre os efeitos farmacodinâmicos da Galantamina são necessários para apoiar a evidência de que ela pode ser usada efetivamente como um tratamento para os sintomas da DA (El-Abhar *et al.*, 2015). Portanto, o objetivo deste estudo foi analisar os efeitos da Galantamina em ratos por meio de um modelo animal da DA induzida por STZ.

Para avaliar os efeitos da Galantamina, primeiramente propomos mimetizar a AD por meio de ICV-STZ em ratos. Então, realizando os testes comportamentais Labirinto Y, Teste de Campo Aberto e Teste de Reconhecimento de Novo Objeto em grupos de animais, é possível medir os efeitos da STZ e do tratamento com Galantamina nos animais. Para analisar as implicações desses fármacos a nível bioquímico, o hipocampo e o córtex pré-frontal dos animais foram analisados pelo método *enzyme-linked immunosorbent assay* (ELISA), a fim de se quantificar as proteínas Aβ e α-Syn.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Investigar os efeitos da Galantamina em um modelo animal de DA induzido por STZ.

2.2 OBJETIVOS ESPECÍFICOS

Mimetizar a DA através de ICV-STZ em uma amostra aletória de ratos

Realizar os testes comportamentais Labirinto em Y, Teste de Campo Aberto e Reconhecimento de Novos Objetos; para medir os efeitos da STZ e implicações do tratamento com a Galantamina.

Analisar o Hipocampo e o cortex pré-frontal através do método ELISA, para quantificar Aβ e α-Syn.

Relacionar os efeitos do tratamento farmacológico com os testes comportamentais e análises bioquímicas.

3 ARTIGO CIENTÍFICO

Effects of Galantamine in Memory Impairment and Increase of β-Amyloid and Alpha-synuclein in Streptozotocin Induced an Animal Model of Alzheimer's Disease.

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ABSTRACT

The Streptozotocin (STZ) induced animal model of Alzheimer's Disease (AD) generates a state of cerebral insulin resistance, capable of mimicking AD by causing oxidative stress mediated by inflammatory process. The levels of inflammatory cytokines can be reduced by Galantamine treatment; thus, its use is indicated to ease AD symptoms, since it is believed that deficiency in glucose metabolism may participate in the pathogenesis of AD. In this context, we aim to investigate the effects of Galantamine regarding memory and locomotion frequency in Wistar rats treated with STZ. To do so, we mimic the AD in male Wistar rats through intracerebroventricular injections of STZ (1mg/kg). Then, behavioral testes were carried for 32 rats: 16 injected with STZ and 16 injected with vehicle. Subsequently, we treated half of the rats in each of the aforementioned groups with Galantamine (5 mg/kg). The other half of the rats of each group were given vehicle. In addition, we performed ELISA to assess Beta Amyloid (Aβ) and Alpha Synuclein (α-Syn) accumulation in the rats' brains. Except for the Novel Object Recognition Test, all other behavioral tests results indicated that the STZ + Vehicle group was impaired when compared to the Sham + Vehicle group. In addition, our results showed no statistically significant differences in the memory of the rats treated with Galantamine, when compared to those only treated with vehicle, regarding behavioral tests. The ELISA showed Aβ and α-Syn accumulation in some groups of rats impaired with STZ. This last result suggests that the locomotion impairment found in the animals could be due to the α-Syn accumulation and not only because of the AD mimicking caused by the STZ. Finally, the animals impaired with STZ did not presented improvements when treated with Galantamine, nonetheless, the overall performance for the STZ + Galantamine group was higher than the STZ + Vehicle one on the NORT. Hence, we believe that more studies on this subject should be carried out to elucidate some challenges yet unresolved.

Keywords: Alzheimer's Disease, Galantamine, Streptozotocin, Alpha Synuclein, Beta Amyloid.

1. Introduction

Dementia is the main cause of progressive disorders in cognitive functions, such as language, memory, rational thinking and social skills, as well as changes in emotion and personality [1]. One of the most common types of dementia is the well-known Alzheimer's disease (AD). Currently, it is estimated that 35.6 million people live with AD and it is believed that this estimate may double every 20 years, reaching 65.7 million in 2030 [2]. As an irreversible and progressive disease AD affects cognition, mainly affecting memory, which leads the individual to total dementia in advanced stages. Nevertheless, the disease can also involve the motor capabilities of those affected by this pathology which makes it the main cause of functional dependence, institutionalization and mortality among the elderly population [2, 3].

The neuropathology of AD follows a pattern similar to other dementias. There a pattern to be followed by neural cells in which the disease develops: neuronal injury, synaptic failure and finally cell death [4, 5]. These findings lead to the two main hypotheses of brain changes caused by AD: the amyloid cascade hypothesis and the cholinergic hypothesis. Both hypotheses suggest that the final outcome of the impairment caused by the AD cascade is the functional loss with the consequent neuronal death, by the accumulation of Beta amyloid protein (Aβ) and TAU tangles [6-8].

In addition, another potential harmful protein is α-synuclein (α-Syn). Its lesions may lead to an unhealthy aging process, through Lewy body, ending up in neurodegenerative disorders such as Parkinson's disease (PD) and Lewy Body Disease [9]. Although α-Syn is commonly considered as a PD biomarker [10], some studies demonstrate that up to 60% of patients with AD have signs of Lewy body disorders [11-14]. In a cohort study with 225 AD patients, it was shown that the α-Syn concentration on the cerebrospinal fluid was significantly increased [15]. Other study suggests that the disturbances of the AD has linkage with simultaneous α-Syn lesions [16]. Moreover, despite the key targets of Aβ and α-Syn are dissimilar, either share mechanisms that leads to oxidative stress [17].

Studies suggest that the decrease in neural glucose metabolism triggers the neurodegenerative cascade of AD [18-20]. From this, Streptozotocin (STZ), a drug

able to induce Insulin Resistant Brain State (IRBS) [21], can be used to mimic some AD clinical features when administered intracerebroventricularly (ICV-STZ). Through low dose injections of STZ into the lateral ventricles of animal's brains, this drug induces oxidative stress [22], neuroinflammation [23], damage to the cerebral cholinergic system [24], formation of positive aggregates for protein Aβ [25] and impairments in cerebral energy metabolism [26]; resulting in cognitive deficits such as impaired memory and spatial orientation [27].

Despite these previously mentioned connections between α-Syn and Aβ regarding the AD, a better understanding of the relationship between AD and α-Syn is needed. Hence, and since to the best of the authors knowledge no study concerning the relation of STZ, Aβ and α-Syn were found in the literature, we decided to investigate this matter a little further in this paper.

Several studies have accumulated evidence that suggests the neuroprotective effect of Galantamine [28-30]. Galantamine has anti-oxidant, anti-apoptotic and anti-inflammatory properties, that way, through its properties it could modulate glucose metabolism [31-33]. The dose commonly used by researchers varies from 1 mg/kg to 5 mg/kg [29, 34-36]. In this scenario, a study found that newborn rats pre-treated with Galantamine have reduced brain damage induced by hypoxia-ischemia. In this study the 5 mg/kg dose of Galantamine showed marked reduction of brain damage when compared to the control group and the 1 and 2.5 mg/kg Galantamine pre-treated groups [34].

However, further studies regarding the pharmacodynamic effects of the Galantamine are required to support the evidence that it can be used effectively as a treatment to the AD symptoms [31]. Therefore, this paper aims to analyze the effects of Galantamine on rats with STZ induced animal model of AD.

To evaluate the Galantamine's effects, first we propose to mimic the AD through ICZ-STZ in the rats. Then, by performing the Y Maze, Open Field Test (OFT) and Novel Object Recognition Test (NORT) on an animal sample, it is possible to measure the STZ and the Galantamine treatment impacts on the animal's performance. Here, the hippocampus and pre-frontal cortex of the animals were also analyzed by enzyme-linked immunosorbent assay (ELISA), to quantify the Aβ and α-Syn.

2. Methods

2.1. Animals

Male Wistar rats, approximately 3 months old, were used at the beginning of the experiments, from the vivarium bioterium of the Biological Sciences Sector of the Federal University of Paraná. The rats were kept in groups of a maximum of 5 per box, in a room with controlled humidity and temperature (22 ± 2 ° C), a 12-hour light-dark cycle, with the light phase starting at 7:00 am. Water and food were provided at will during all experiments.

All procedures were conducted according to the ethics and research protocols of the Federal University of Paraná and this research was approved by the university's ethics committee, with the number 1226.

2.2. Drugs

- Streptozotocin 1 mg/kg (STZ) was purchased from Santa Cruz Biotechnology Inc., Santa Cruz, USA.
- Galantamine 5 mg/kg was purchased from Prati, Donaduzzi & Cia Ltda., Toledo, Brazil.

2.3. Experimental design

Thirty-two rats were randomly divided into groups sham (n = 16) and STZ (n = 16). STZ group received bilateral injection of STZ, at a dose of 1 mg/kg, dissolved in 0.9% sterile saline solution in the lateral ventricles. The sham group received only sterile saline. Daily administrations of Galantamine were performed to assess the effects of this treatment in STZ injured animals, on cognitive functions, spatial memory, locomotion frequency and short-term recognition. For such treatment, the sham and STZ groups were randomly subdivided into 4 groups of 8 animals each: Sham + Vehicle; Sham + Galantamine; STZ + Vehicle and STZ + Galantamine (Figure 1).

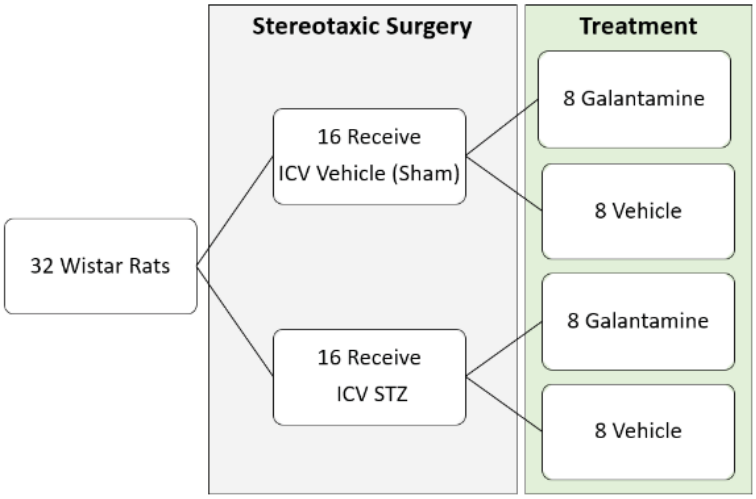


Figure 1 - *Groups, procedures and number of animals per group. ICV: Intracerebroventricular; ICV-STZ: Intracerebroventricular Streptozotocin.*

Galantamine was administered to the animals for 30 days intraperitoneally. It started 1h before stereotaxic surgery, using 0.5% carboxymethylcellulose in distilled water with 1% Tween 80 as a vehicle. Galantamine was able to produce results in Rats in doses of 4 mg/kg or 5 mg/kg [36]. In this scenario, the dose of Galantamine used in this paper was 5 mg/kg. Behavioral tests were performed on the animals:

- On the 21st day after surgery, the OFT was performed.
- On the 28th day the rats went through the habituation of the box of objects and then on the 29th day they performed the object location test.
- On the 30th day, the Y Maze test was fulfilled.

At the end of the behavioral tests, on the 30th day of the experiment, the animals were euthanized and the brains dissected for measurement of β-amyloid protein and alpha synuclein in the hippocampus and prefrontal cortex.

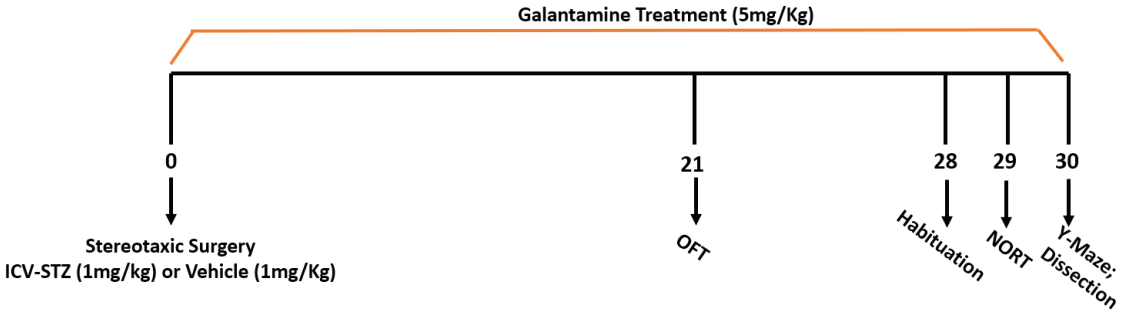


Figure 2 – *Experimental design. Scheme of procedures and tests performed. ICV: Intracerebroventricular; ICV-STZ: Intracerebroventricular Streptozotocin OFT: Open Field Test; NORT: Novel Object Recognition Test.*

2.4. Stereotaxic surgery

Before stereotaxic surgery, the animals were anesthetized with Equitesin 0.3mg/kg (distilled water, chloral hydrate, magnesium sulfate, 90% alcohol, thiopental and propylene glycol). Thereafter, they received atropine sulfate (0.4 mg / kg, i.p.). After this procedure, the animals were placed in a stereotaxic (David Kopf, model 957L). Through a scalpel, access to the skull was established and for the location of the ventricles, the following coordinates were adjusted: anteroposterior -0.8 mm, lateral 1.5 mm and dorsoventral 4.0 mm relative to the bregma and ventral from the dura with the bar set to 0 mm [37]. After craniotomy, STZ was infused with a 30-gauge needle connected to a polyethylene tube connected to a 10μl micro-syringe (Hamilton, USA). The composition of this equipment was linked to an infusion pump (Havard Apparatus, USA). In the STZ groups, 1mg / kg of STZ (5 μl per injection site) was infused into the lateral ventricles. In the sham groups, the same methodology was used, however, the rats were treated with sterile saline solution, instead of STZ.

At the end of the surgeries, the rats were transferred to individual boxes in a heated room, where they were able to recover from the procedure and only then could be transported to vivarium.

2.5. Behavioral Evaluation

2.5.1 Open Field Test

On the 21st day after the surgery, the OFT was performed to analyze spontaneous locomotor activity. The OFT consists of a circular arena (97-cm diameter, 42-cm height) separated into three concentric circles and subdivided into 19 quadrants. Each animal was allocated in the center of the equipment where it could freely explore the arena for 5 minutes. From this test, the total number of crossings from one quadrant to another (number of line crossings) was measured. A crossing was considered only when all the 4 rat's paws were within the same quadrant. The objects and the box were cleaned with 10% alcohol in order to avoid olfactory bias between each animal; all assays took place in a controlled lighting room (20 lx).

2.5.2 Y Maze Test

The Y Maze test was performed on the 29th day after surgery as previously described by Bassani, Turnes [38]. Using this test it was possible to assess short term spatial memory [39, 40]. The Y Maze consists of an apparatus in the shape of the letter Y, made of wood and painted black, with three arms separated by 120º angles, measuring 50 cm in length, 12 cm in width and 27 cm in height. The animals' behavior was filmed for later evaluation with a camera. In the training session of the Y Maze, one of the arms was unreachable by a wooden door located in front of it. The rats were placed in one of the other two arms (initial arm), and were able to explore these maze arms for 5 min. After 1h of the training session it was performed the Y Maze test itself. The blockade was detached, allowing access to all of the arms. The previously blocked arm was named “new arm”. Rats were placed back in the initial arm and were able to freely explore for 3 min. To evaluate the short-term spatial memory, was considered the percentage of time exploring the new arm, regarding the total exploratory time [41]. The maze was cleaned with 10% alcohol between each session and each animal to reduce olfactory bias; all assays took place in a controlled lighting room (20 lx).

2.5.3 NORT

The NORT was performed in order to assess short-term memory [42]. The percentage of exploration of the new object was measured, in relation to the total exploratory time of the two objects [43]. The environment and gears used were standardized by Bassani et al (2017). Test and training (habituation) took place in a square box (100 x 100 x 40 cm), made of wood. A camera was positioned to obtain an aerial view of the arena to record the animals' behavior, for later evaluation. Before NORT was performed, first the animals went through a habituation stage on the 28th day after the surgery, where each animal was placed in the empty box for 5 minutes of free exploration. After that, a new 5 minutes habituation session was held 24h later, on this session 2 identical objects were positioned symmetrically in the apparatus, approximately 10 cm away from the walls. The animals were able to explore the objects for 5 min and then were returned to their housing boxes. Subsequently the second training session a third one was performed, identical to the previous session, respecting an 1h interval. After 1h of the training sessions, the NORT itself was

implemented (29th day after surgery). On NORT one of the objects was kept (familiar object) and the other one was replaced by a different object (new object). Both remained in their original positions. Each animal was placed in the arena for 3 minutes to explore the objects [44]. In order to avoid olfactory bias, before each session and between each animal, the objects and the box were cleaned with 10% alcohol; all assays took place in a controlled lighting room (20 lx).

2.6. Neurochemical Analysis

At the 30th day after surgery, the brains were rapidly extracted on dry ice, and the whole hippocampus and prefrontal cortex were dissected and stored at −80 °C. The tissue homogenate of these biological samples was prepared, according to the manufacturer's instructions (USCN Business Co., Wuhan, Hubei, China) and Enzyme-Linked Immunosorbent Assay (ELISA) was made for Aβ and α-Syn. The tissues were washed in ice-cold phosphate buffered saline (PBS) to remove blood excess and weighed before homogenization. After that, the samples were minced and homogenized in fresh lysis buffer (complete™, EDTA-free Protease Inhibitor Cocktail from Roche). The resulting suspension was sonicated with an ultrasonic cell disrupter to clarification. After that the homogenates were centrifuged for 5 minutes at 10.000×g and then was collect the supernatant to perform the assay. Subsequently, was performed the quantification of Aβ (product No. CEA946Ra) and α-Syn (product No. SEB222Ra) of these samples by ELISA according to the manufacturer's instructions (USCN Business Co., Wuhan, Hubei, China). The assays were performed as duplicates and the means of the microplate reader were used (measured at 450 nm), its results are expressed as absorbance.

2.7. Statistical Analysis

Data was presented as mean ± standard error of mean (SEM). The Kolmogorov-Smirnov test was used to confirm the data was normally distributed. In order to assess the influence of both factors (Galantamine and STZ) on all response variables (behavioral tests and ELISA), as well as factors interactions, difference between groups were assessed performing a two-way Analysis of Variance (ANOVA) with significance level equal to 5% ($\alpha = 0.05$).

Thus, in this paper, when the ANOVA test showed a Fisher's p-value (p) less than the significance level for any source of variation, i.e. $p < 0.05$, Tukey's multiple comparisons tests were made to investigate means differences between each group. Here, again, a significant difference was considered when the $p < 0.05$.

3. Results

3.1. Locomotion Frequency Test

On the OFT it was measured the spontaneous locomotor performance activity by accessing the total number of crossings (Figure 3). The ANOVA test for the OFT only showed statistically significance difference for the STZ effect ($p = 0.0001$). The Tukey test shows statistically significant difference between Sham + Galantamine and STZ + Vehicle groups (p = 0.0017). There is also statistically significant difference between Sham + Galantamine and STZ + Galantamine groups (p = 0.0050).

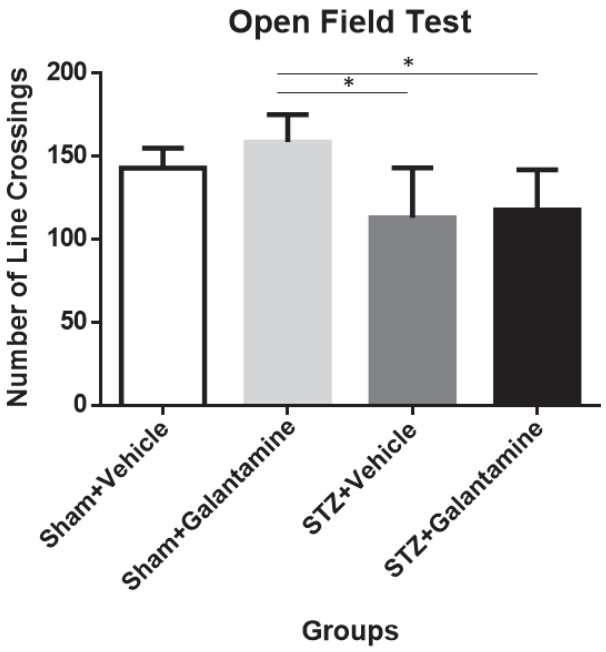


Figure 3 – *Effects of prolonged Galantamine (5 mg/kg) in spontaneous locomotor activity of Sham and STZ groups. All data is expressed as mean ± SEM. *p < 0.05, indicating a statistically significant difference between groups. (ANOVA). N = 8 animals per group.*

3.2. Memory Tests

Through the Y Maze test it was possible to assess the short-term spatial memory, by measuring the percentage of time exploring the new arm (Figure 4).

The ANOVA test for the Y Maze only showed statistically significance difference for the STZ effect ($p = 0.0041$). Multiple comparison Tukey's test showed a statistically significant difference between the Sham + Vehicle and STZ + Vehicle groups (p = 0.0218).

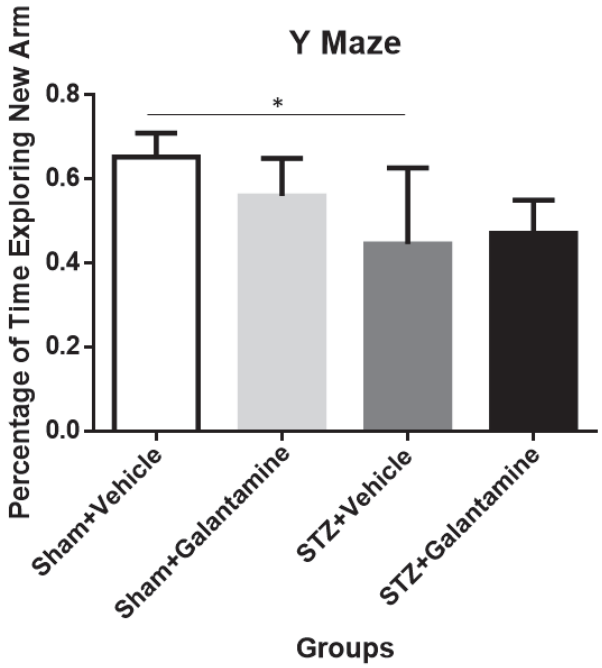


Figure 4 – *Prolonged Galantamine administration (5 mg/kg) and its impact in the short-term spatial memory. All data are expressed as mean ± SEM. *p < 0.05, indicating a statistically significant difference between groups. (ANOVA). N = 8 animals per group.*

No effect of treatment was seen in the percentage of exploratory time of the new object (Figure 5).

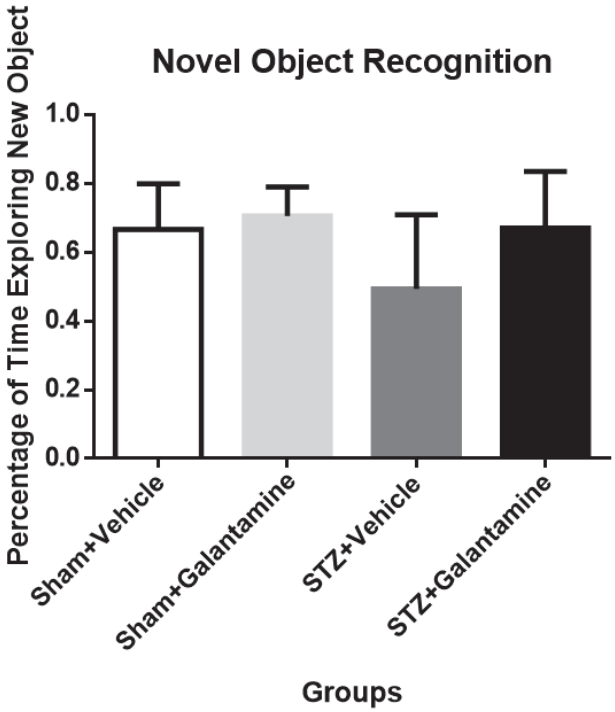
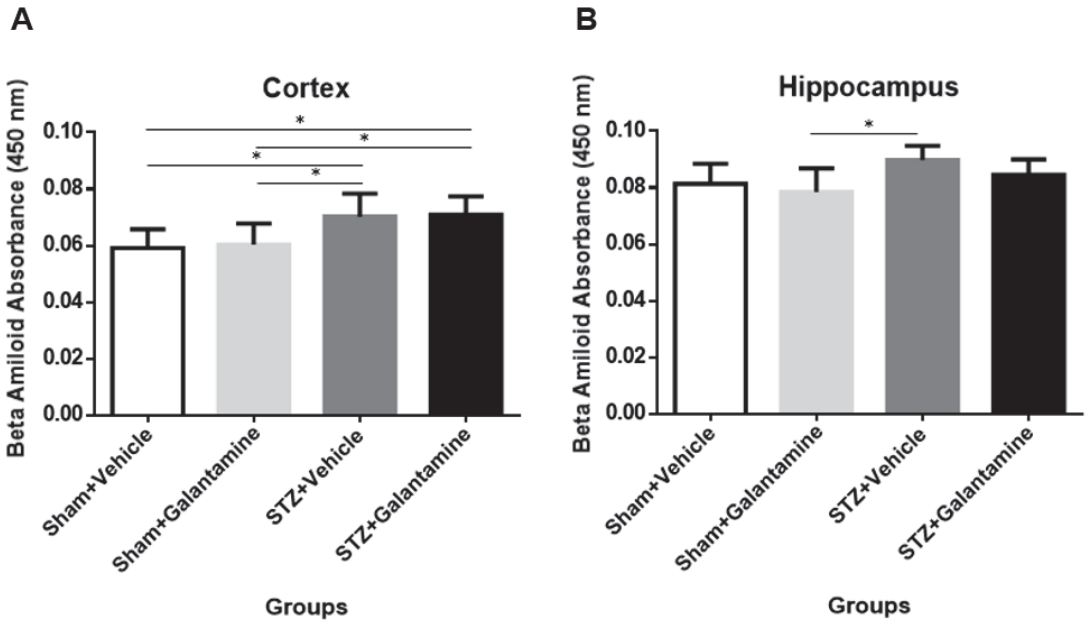


Figure 5 – *Short-term memory effects of prolonged Galantamine (5 mg/kg) administration in Sham and STZ groups. The data are expressed as mean ± SEM. N = 8 animals per group. There was no statistically significant difference between groups (ANOVA).*

3.3. Quantification of beta amyloid and alpha synuclein by enzyme-linked immunosorbent assay (ELISA)

The accumulation of Aβ in the prefrontal cortex and in the hippocampus showed an increase in this protein in the STZ-lesioned rats ($p = 0.0002$ and $p = 0.0048$ respectively). In cortex both STZ groups presented higher Aβ absorbance than the Sham groups (Figure 6A). Regarding the Galantamine treatment, there was no difference between STZ + Vehicle and STZ+ Galantamine. In hippocampus there was more accumulation of Aβ in the STZ groups but the STZ + Galantamine group had less Aβ concentration, when compared to the STZ + Vehicle group. The only statistically significant difference observed in Figure 6B is between the Sham + Galantamine and STZ + Vehicle groups (p = 0.0112).

Figure 6 - *Quantification of Aβ levels in the prefrontal cortex (**A**) and hippocampus (**B**) by ELISA. Statistical relationship between animals injured with STZ and Sham. It is also shown the implications of prolonged use of Galantamine (5 mg/kg) in either group. All data is expressed as mean ± SEM. *p < 0.05, indicating a statistically significant difference between groups. (ANOVA). N = 8 animals per group.*



When it comes to α-Syn accumulation, it is observed an accumulation of this protein in the cortex of the STZ groups (Figure 7A). Excluding the statistical difference between Sham + Galantamine and STZ + Vehicle, Galantamine had no impact in the accretion of this protein, regarding cortex.

The phenomenon of more α-Syn accumulation in the STZ groups is not observed in the hippocampus (Figure 7B), however, when observing the galantamine effect, it is notice less α-Syn in the STZ + Galantamine group when compared to the STZ + Vehicle group. Moreover, it is observed a statistical difference between the Sham + Galantamine and STZ + Vehicle groups: p = 0.0268.

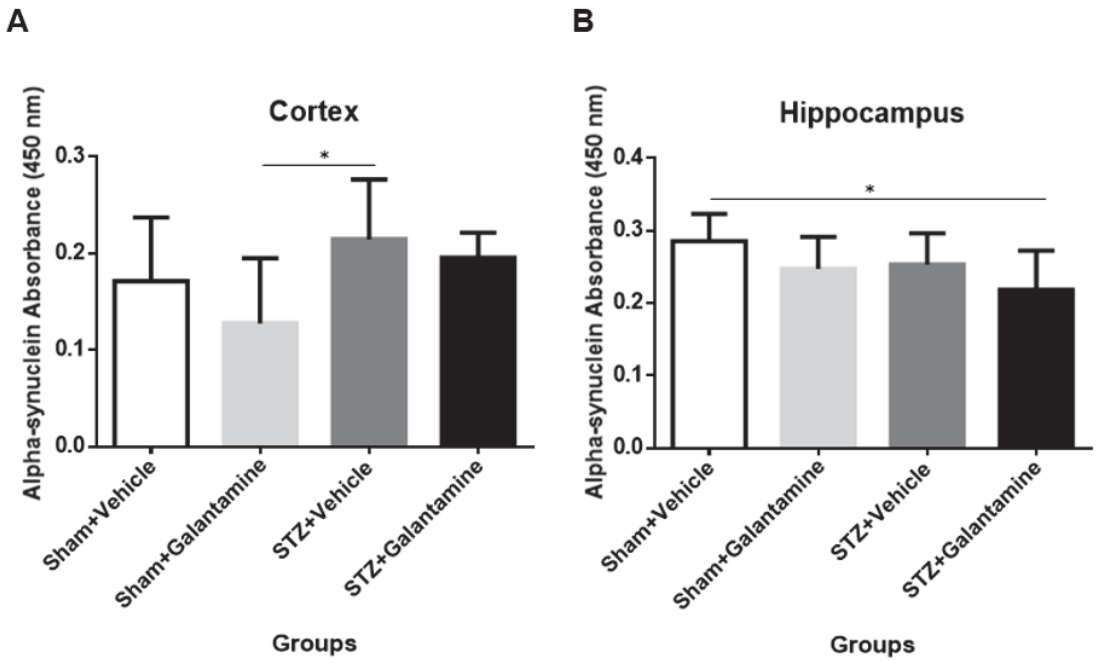


Figure 7 - *Quantification of* α-Syn *levels in the prefrontal cortex (**A**) and hippocampus (**B**) by ELISA. Statistical relationship between animals injured with STZ and Sham. It is also shown the implications of prolonged use of Galantamine (5 mg/kg) in either group. All data is expressed as mean ± SEM.* $*p < 0.05$*, indicating a statistically significant difference between groups. (ANOVA). N = 8 animals per group.*

4. Discussion

The present study investigated the STZ-lesion in galantamine treated rats. The results showed that STZ groups had reduced locomotion frequency in addition to a lesser percentage of time exploring the new arm in Y maze test. we found that the STZ increased the accumulation of Aβ in the prefrontal cortex as well as in the hippocampus. Moreover, the STZ-lesioned rats exhibited an increase in the α-Syn in the prefrontal cortex. The Galantamine treatment did not present signs of reversal in these effects.

The OFT and Y Maze test showed significant differences in the locomotion frequency and memory of the animals (Figure 3). The performance of the animals in the STZ + Vehicle and STZ + Galantamine groups are similar, indicating that the Galantamine had no effect on the locomotion frequency. Furthermore it is shown that both the Sham groups presented better performance than the STZ groups on the OFT. These results show that the injured animals had a diminished locomotion frequency.

Moreover, as can be seen by the results of the Y Maze test (Figure 4), the mean percentage of time exploring the new arm by the Sham + Vehicle group was significantly higher than the STZ + Vehicle group. One can notice that all STZ groups presented lower memory performance in this test. This result gives support to the AD's animal model, since the short-term spatial memory on STZ + Vehicle is damaged when compared to the Sham + Vehicle group. Furthermore, there was no statistically significant difference between groups treated with the Galantamine.

Regarding the NORT results, it was observed (Figure 5) there were no statistically significant differences between any of the groups. Nonetheless, even without statistical significance, the STZ + Vehicle group had diminished memory performance when compared to all other groups. In addition, the STZ + Galantamine group presented better performance than the STZ + Vehicle group. These results may indicate that the Galantamine could ease the AD symptoms. However, more researches are needed to further investigate this hypothesis.

Since in the Novel Object Recognition behavioral test there were no statistically significant differences between Sham and STZ groups, one could suggest that the STZ animals were not in an advanced AD stage. One possible explanation to this result could be related to the low dose of STZ. What gives substantial support to this hypothesis is that the spatial memory is one of the first types of memory affected by the AD, with some authors even suggesting that it may occur in preclinical stages [45]. In this regard, our results indicate that the STZ group presented impairments on its spatial memory performance, since these animals did not perform well on the Y Maze test (Figure 4).

In this scenario, we suggest that memory and locomotor performances were diminished due to the same trigger mechanism: the IRBS and oxidative stress, since STZ is a drug capable of inducing glucose metabolism impairments through its affinity with GLUT-2 [18]. In this connection, some authors even suggest that AD may be a type 3 diabetes [46, 47].

However, no statistically significant difference regarding memory and locomotion improvement due to the Galantamine treatment was found on the animals impaired by the STZ. Possibly the lack of the Galantamine effect observed is this paper is due to its action mechanism and the temporal window in which this drug is able to produce beneficial outcomes. Galantamine is a selective, competitive and reversible acetylcholesterase inhibitor.

In addition, it is able to increase the intrinsic action of acetylcholine on nicotinic receptors (modulation), probably through binding to an allosteric site of this receptor [48]. As consequence of the improvement in the cholinergic system, a boost in the cognitive functions of AD patients can be observed [49].

Although acetylcholinesterase inhibitors can temporarily attenuate memory and cognitive impairments in AD patients [49], AD is a progressive disease and currently there is no treatment regarding the ongoing cellular loss [48].

In this line, in 2012, a study in Guinea pigs showed that Galantamine prevents cell death due to apoptosis induced by Soman 28 μg/kg, an anticholinesterase drug [50]. Also, there is indicatives that there is a link regarding the basal forebrain cholinergic system and the AD pathogenesis [51]. Since Galantamine is a reversible acetylcholinesterase inhibitor, being an activator of cholinergic-inflammatory pathway by suppressing levels of TNF and IL-6 [30, 35, 52-54], is supposed that this drug is able to improve the inflammatory cytokines response, consequently interfering in the Aβ formation cascade [55].

In addition, the Galantamine presents disease-modifying capacities, as has been reported in Van Dam and De Deyn [56], and references therein, in this scenario, we suspect that the insufficient performance of the Galantamine in our study is linked with the preventive characteristics of this drug. Perhaps a more prolonged treatment before stereotaxic surgery [57], combined with a different dosage schedule aiming at the preventive and disease-modifying characteristics, would have found a different outcome for the results shown in this paper.

The lesser frequency locomotion found in the rats could be due to the α-Syn accumulation and not only because of the AD animal model induced by STZ. In addition, another noticeable result is that in most of the tests there were no statistically significant differences in the memory of the rats treated with Galantamine, when compared to those only treated with vehicle. However, one cannot assume promptly that Galantamine has no effect whatsoever in treating AD clinical features, since some differences were observed.

Regarding the Aβ accumulation measured by the ELISA (Figure 6), the STZ + Galantamine group presented a lower absorbance in the prefrontal cortex, when compared to the Sham + Vehicle group (Figure 6A). This result indicates that the STZ reproduces a feature of AD, which was an expected result since this type of AD animal

model was already described by Bassani et al. (2017). Moreover, other differences point to the fact that Galantamine has no effect mitigating the neuroinflammatory cascade, since both STZ groups presented higher Aβ absorbance in the prefrontal cortex than the Sham groups, as can be seen in Figure 6A.

The results observed for the prefrontal cortex also support the AD STZ animal model (Figure 6B), showing that the STZ impaired rats presented higher Aβ absorbance than the Sham ones. There was statistically significant difference between the Sham + Galantamine and STZ + Vehicle groups, due to the STZ effect, as shown by the two-way ANOVA result ($p = 0.0048$).

The results of the ELISA with respect to α-Syn absorbance (Figure 7) indicates that not only the animals had AD biomarkers, but also presented what is usually considered an exclusive PD biomarker [58]. The high absorbance of the α-Syn in the prefrontal cortex, seen on Figure 7A, may be the cause of the performance impairment seen on the OFT, indicating that the rats could be suffering from parkinsonism.

It is well-known that, in contrast to the AD, PD is characterized by motor impairment caused by accumulation of α-Syn in dopaminergic neurons, forming structures called Lewy bodies [59]. In addition, in its late stages, PD may cause cognitive deficits and dementia [60-62]. Despite one can find different protein accumulation in the neural tissue of AD and PD patients brains, Aβ and α-Syn, respectively, both diseases may have common pathological triggers, like neuroinflammation and oxidative stress [63], as well as both proteins accumulation [16, 64]. Thus, some scientists believe that PD and AD may have a common metabolic physiopathology [21].

Hence, although one can suggest that PD and AD may have common trigger mechanisms, such as oxidative stress [17], the fact that in this paper was found higher α-Syn absorbance in the STZ lesioned rats, it is yet not enough to confirm that the rats have mimicked the PD thoroughly. We believe that the animals have presented motor impairment due to the α-Syn accumulation. This statement could be accepted, since some studies demonstrate that up to 60% of patients with AD also present signs of Lewy body disorders [11-14].

Regarding the difference in α-Syn accumulation in the Hippocampus between the Sham + Vehicle and STZ + Galantamine groups, the two-way ANOVA indicated that the variation between groups was due to the Galantamine effect ($p = 0.0292$). Hence,

the phenomenon described in the previous paragraph could explain the results shown on Figure 7B. The lower absorbance of α-Syn in the impaired group due to the Galantamine effect indicates that the drug could ease the IRBS and its damages.

Finally, the groups treated with STZ presented both Aβ and α-Syn accumulation, indicating that the high absorbance of these proteins may have a common source.

Nevertheless, since, to the best of the authors knowledge, this is one of the first studies concerning Galantamine effects on the AD animal model induced by STZ, as well as on the assessment of α-Syn accumulation, we believe that our results cheer more researches in this direction.

5. Concluding remarks

This paper presented the Galantamine effects on a STZ AD animal model. Despite the fact that the animals impaired with STZ did not presented improvements when treated with Galantamine, the overall performance for the STZ + Galantamine group was higher than the STZ + Vehicle one on the NORT. On the other hand, on the ELISA all the STZ groups had higher α-Syn and Aβ accumulation in their Hippocampus and Prefrontal Cortex. One result (Figure 7B) indicated that Galantamine may have diminished the α-Syn accumulation of the STZ impaired group.

The locomotion impairment found in the animals is due to the α-Syn accumulation, and not because of the AD. STZ animals were not in an advanced AD stage, the impairment could be due to the IRBS, which can cause either α-Syn accumulation and memory impairment (AD). Nevertheless, we believe that more studies on this subject should be carried out to elucidate some challenges yet unresolved.

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4 CONSIDERAÇÕES FINAIS

Correspondendo de 60% a 80% dos quadros demenciais (Götz *et al.*, 2018), a DA ainda representa um grande desafio tanto do ponto de vista do entendimento da doença, quanto do seu tratamento. Esses dois fatores devem servir como estímulo a mais pesquisas nessa linha, pois, atualmente, é uma das patologias mais temidas, que carece de terapêutica modificadora da doença, bem como curativa. A presente tese demonstrou a possibilidade da DA possuir fatores fisiopatológicos em comum com a DP, o que demonstra que nem ao menos a completa história natural da doença dominamos. Diante desse quadro é fácil entender o porquê de ainda não existirem tratamentos eficazes, que curem e ou modifiquem os sombrios desfechos clínicos encontrados na DA.

O modelo animal da DA pela STZ se mostrou eficaz para avaliar os efeitos da Galantamina, produzindo características clínicas e bioquímicas condizentes com a doença. Ainda não existindo consenso sobre os gatilhos iniciadores da formação da Aβ, as crescentes evidências da presença de α-Syn despertam ainda mais enigmas sobre a DA, ao mesmo tempo que proporcionam potenciais alvos terapêuticos para a intervenção nessa patologia.

Embora nesse trabalho a Galantamina tenha demonstrado efeitos menores nos sinais correspondentes à DA nos animais. Esse fármaco apresenta relativamente bons resultados no manejo clínico de pacientes com DA moderada a grave. Enquanto nenhuma outra terapia com eficácia comparável ou melhor estiver disponível, a Galantamina ainda permanecerá como uma droga de primeira escolha para o tratamento da DA (Prvulovic *et al.*, 2010).

5 CONCLUSÃO

O artigo dessa tese demonstrou efeitos da Galantamina em um modelo animal de DA induzido por STZ. Apesar de os animais lesados pela STZ não apresentarem melhora quando tratados com Galantamina, o desempenho do grupo STZ + Galantamina foi superior ao do grupo STZ + Veículo no Teste de Reconhecimento de Novo Objeto.

Todos os grupos STZ apresentaram maior acúmulo de α-Syn e Aβ em seu Hipocampo e córtex pré-frontal. No Hipocampo, a Galantamina pode ter diminuído o acúmulo de α-Syn nos ratos lesados pela STZ.

Acreditamos que o comprometimento da locomoção encontrado nos animais é decorrente do acúmulo de α-Syn e não apenas de Aβ, indicando que a DA pode ser mais complexa do que o modelo clássico que apenas considera o acúmulo de Aβ como fator patognomônico.

Sugerimos que os animais lesados pela STZ não estavam em estágio avançado da DA, uma vez que não houveram diferenças estatisticamente significantes entre os grupos no Teste de Reconhecimento de Novo Objeto.

Por fim, propomos que a DA e a DP podem ter origem comum, o estado cerebral de resistência à insulina, pelo fato de os grupos tratados com STZ terem apresentato acúmulo tanto de Aβ quanto de α-Syn.

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